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The small heat shock-related protein–20 is an actin-associated protein

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Purpose: **The activation of cyclic nucleotide-dependent signaling pathways in vascular smooth muscle is important for the prevention of vein graft spasm and neointimal hyperplasia. Cyclic nucleotide-dependent relaxation is associated with an increase in the phosphorylation of a small heat shock-related protein (HSP20). In this investigation, we examined the mechanisms by which HSP20 may modulate relaxation.**
Methods: **The relaxation responses of the bovine carotid artery smooth muscles were determined in a muscle bath. HSP20 phosphorylation was quantitated with isoelectric-focusing immunoblots. The association with actin was determined with coimmunoprecipitation and cosedimentation. Molecular sieving columns were used to examine the macromolecular associations of HSP20.**
Results: **The activation of cyclic nucleotide signaling pathways leads to the complete relaxation of carotid smooth muscle. This relaxation response is associated with an increase in the phosphorylation of HSP20. Actin coimmunoprecipitated with HSP20, and the association of actin with recombinant HSP20 in vitro was phosphorylation-state dependent. Finally, HSP20 exists in large (>100 kDa) aggregates, which dissociate with the activation of cyclic nucleotide signaling pathways.**
Conclusion: **These data support a role of HSP20 phosphorylation in mediating smooth muscle relaxation, possibly via a direct interaction of large aggregates of HSP20 with the contractile elements. (J Vasc Surg 1999;29:326-33.)**

Human saphenous vein is a well-established coronary and peripheral arterial bypass graft conduit. However, the human saphenous vein is prone to vasospasm, which may contribute to graft failure in aortocoronary and peripheral vascular reconstructions.[1-4] Treatment with papaverine, a phosphodiesterase inhibitor that prevents the breakdown of the cyclic nucleotides, cyclic adenosine monophosphate (cAMP), and cyclic guanosine monophosphate (cGMP), has been recommended to prevent vein graft spasm during arterial reconstructions.[1]

Smooth muscle migration and proliferation have been implicated in the pathogenesis of atherosclerosis and intimal hyperplasia.[5] The activation of the cGMP pathway has been shown to inhibit vascular smooth muscle proliferation in vitro and in vivo.[6-8] In addition, the activation of the cGMP pathway inhibits angiotensin II–stimulated migration of smooth muscle cells.[9] Thus, the activation of cyclic nucleotide-dependent signaling pathways in vascular smooth muscles may be important in the prevention of vein graft spasm and vascular remodeling processes that lead to both short-term and long-term vein graft failure.

Nitric oxide and nitric oxide donors, such as sodium nitroprusside, relax smooth muscle by activating guanylate cyclase, which leads to increases in intracellular cGMP.[10] Other vasodilators, such as prostacyclin

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and isoproterenol, interact with cell surface receptors, which leads to the activation of adenylate cyclase with an increase in cAMP.[11] Increases in cGMP and cAMP lead to the activation of cGMP-dependent protein kinase (PKG) and cAMP-dependent protein kinase (PKA), respectively.[10,11] We recently have demonstrated that the activation of either the cAMP/PKA or the cGMP/PKG pathway leads to a final common event—an increase in the phosphorylation of a small heat shock protein (HSP20).[12]

Together, these findings suggest that the activation of the cGMP/PKG pathway, which leads to increases in the phosphorylation of HSP20, may prevent pathologic functional (vasospasm) and structural (atherosclerosis/intimal hyperplasia) narrowing of blood vessels. Thus, the elucidation of the mechanisms by which HSP20 modulates smooth muscle cell physiology can lead to significant advances in our understanding of vascular smooth muscle biology and can target therapeutic approaches to pathologic alterations that occur in diseases, such as vasospasm and intimal hyperplasia.

METHODS

Materials. Electrophoresis reagents and the DC protein assay kit were from Biorad (Hercules, Calif). The rabbit anti-HSP20 antibodies from Kato et al[13] and the anti–smooth muscle α-actin antibodies (A-2547) were from Sigma Chemical Corp (St Louis, Mo). The catalytic subunit of cAMP-dependent PKA was from Promega (Madison, Wis). The forskolin, the N-2-hydroxyethylpiperazine-N'-2-ethane-sulfonic acid, the ethylenediaminetetra-acetic acid (EDTA), the ethylene glycol-bis(β-aminoethyl ether)-N,N,N',N'-tetra-acetic acid, and the (3-([3-cholamidopropyl]dimethylammonio)-1-propanesulfonate (CHAPS) were from Sigma Chemical Corp. All the other reagents were of analytic grade.

Contractile physiology. Bovine calf carotid arteries were harvested at a local abattoir and dissected free of adventitial tissue. The arteries were opened longitudinally, and the endothelium was denuded by rubbing the intima with a cotton-tipped applicator. Transverse sections of 1 mm were cut and mounted in a tissue bath with silk suture. One end was anchored to a fixed glass pipette, and the other to a force transducer (Grass Instrument Corp, Quincy, Mass). Tension was recorded on a strip-chart recorder (Gould Instrument Corp, Norcross, Ga). The strips were equilibrated for 60 minutes at 37°C in water jacketed chambers that contained Krebs-bicarbonate buffer (120 mmol/L NaCl, 4.7 mmol/L KCl, 1.0 mmol/L $MgSO_4$, 1.0 mmol/L NaH_2PO_4, 10 mmol/L glucose, 1.5 mmol/L $CaCl_2$, and 25 mmol/L Na_2HCO_3). The strips were gassed with 95% O_2/5% CO_2 and maintained at pH 7.4. The maximal contractile length was adjusted with repeated additions of 110 mmol/L KCl, with equimolar replacement of NaCl. All the data were reported as percentages of the maximal response to 110 mmol/L KCl. The agonists and inhibitors were added directly to the muscle bath.

Isoelectric-focusing immunoblots. Protein (30 μg) was loaded onto a 12 × 15–cm slab of isofocusing gels that consisted of 4% acrylamide, 0.1% piperazine diacrylamide, 9 mol/L urea, 5% ampholine (5 parts 6-8, 3 parts 5-7, and 2 parts 3-10), and 2% CHAPS. The cathode buffer consisted of 20 mmol/L sodium hydroxide, and the anode buffer of 10 mmol/L phosphoric acid. The proteins were focused for 10,000 V/h. The gels were equilibrated for 30 minutes in 10 mmol/L Tris pH 6.8, 3% sodium dodecylsulfate (SDS), 19% ethanol, and 4% 2-mercaptoethanol. The gels then were transferred to Immobilon (Millipore Corp, Bedford, Mass) 100 mAmp for 12 hours. The blots were fixed with 20% methanol, dried, blocked with TBS (10 mmol/L Tris, 150 mmol/L NaCl, 0.5% Tween-20, pH 7.4) and 5% milk for 1 hour, washed three times with TBS, and then probed with anti-HSP20 antibodies (1:5000 dilution in TBS, 5% milk) for 1 hour. The blots were washed six times with TBS and 0.5% Tween-20 and then were probed with Supersignal Chemiluminescent Substrate (Pierce, Rockford, Ill) and exposed to film.

Immunoprecipitation. Strips of bovine carotid artery smooth muscle were homogenized in TBS (0.5 g of tissue per mL of buffer), and then the samples were centrifuged at 10,000*g* for 15 minutes. The soluble proteins then were diluted 10-fold with TBS. The anti-HSP20 antiserum was added to the supernatants (1:50 dilution). The samples were shaken gently for 14 hours at 4°C. Protein A-sepharose beads (1/10 volume) were added, and the samples were incubated for an additional 3 hours at 4°C. The beads were washed six times with TBS and 0.5% Tween-20. A final wash of 10 mmol/L Tris pH 7.4 then was done. The proteins were separated on 10% SDS-polyacrylamide gel electrophoresis (SDS-PAGE) gels, transferred to Immobilon, and probed as described previously for isoelectric-focusing immunoblots. The smooth muscle α-actin was used at a dilution of 1:1000.

Cloning and expression of HSP20. The rat complementary DNA for HSP20 (from Dr Kato[14]) was amplified with phosphocreatinine (PCR) with sense (GAA TTC ATA TGG AGA TCC GGG TGC CTG TGC) and antisense (CGT ACT CGA GCT ACT TGG CAG CAG GTG GTG ACT) primers that

were synthesized by Gibco BRL (Grand Island, NY). The PCR products were ligated into PCR-script SK (+) cloning vector and transformed into *Escherichia coli* (*E coli*) supercompetent cells according to the manufacturer's directions (PCR-Script SK (+) Cloning Kit, Stratagene, La Jolla, Calif). The transformed *E coli* was plated onto agar plates, which contained ampicillin, methicillin, 5-bromo-4-chloro-3-indoyl-β—D-galactopyranoside, and isopropyl-β—D-thiogalactopyranoside. The appropriate colonies were selected and placed overnight in an LB-Amp broth at 37°C. Plasmid minipreps were performed with the Wizard miniprep DNA purification system (Promega, Madison, Wis). The isolated DNA then was sequenced with an ABI Prism automatic DNA sequencer (Applied Biosystems, Los Angeles, Calif). The plasmid then was cut with Xho-1, isolated on a 1% agarose gel, and inserted into a pET-19b plasmid. The plasmid was transformed into *E coli* JM109 cells (Promega, Madison, Wis), and the plasmid preparations were performed as previously mentioned. The plasmids then were transformed into BL21(DE3)pLysS cells, which were inoculated onto agar plates. The colonies then were inoculated into LB broth, which contained carbenicillin and chloramphenicol, and grown for approximately 2.5 hours at 37°C until the optical density was 600Au. Isopropyl-β—D-thiogalactopyranoside (1 mmol/L) was added for 30", followed by rifampicin for 90". The bacteria were harvested by means of centrifugation at 2500*g* for 10 minutes, and the HSP20 was affinity purified with an HIS-bind resin column (Novagen, Madison, Wis). Briefly, the pellets were sonicated in 1× binding buffer, and the suspension then was centrifuged at 20,000*g* for 15 minutes. The pellet was resuspended in 5 mL binding buffer/6 mol/L urea, sonicated, and incubated on ice for 1 hour. The suspension was centrifuged at 39,000*g* for 20 minutes, and the supernatant was filtered through a 45-μm filter. The supernatant was applied to the HIS-bind resin column and eluted with the manufacturer's buffer. Proteins from the fractions were separated on SDS-PAGE gels, and the fractions that contained a single band at 20 kDa underwent dialysis against decreasing concentrations of urea (6 mol/L urea/1% triton to 0 mol/L urea/1% triton) in PBS. Finally, the HSP20 underwent dialysis against PBS/1% CHAPS.

In vitro phosphorylation of recombinant HSP20. Recombinant HSP20 (50 μmol/L) was phosphorylated in a reaction mixture, which contained 500 μmol/L adenosine triphosphate, 1 mmol/L $MgCl_2$, and 300 nmol/L of the catalytic subunit of cAMP-dependent PKA, in PBS for 30 minutes at room temperature. The reaction was stopped with the addition of 100 μmol/L of the cAMP-dependent PKA inhibitor. Nonphosphorylated HSP20 was treated with the same reaction mixture without the cAMP-dependent PKA.

Actin cosedimentation assay. Recombinant HSP20 was phosphorylated in vitro with the catalytic subunit of PKA, and the reactions were stopped with the addition of the peptide inhibitor of PKA. Phosphorylated and nonphosphorylated HSP20 (10 μmol/L) were added to a reaction mixture, which contained 10 μmol/L actin, 20 mmol/L Tris-HCL, pH 7.5, 0.1 mol/L KCl, 2 mmol/L $MgCl_2$, 1 mmol/L adenosine triphosphate, 1 mmol/L dithiothreitol, and 0.1 mmol/L $CaCl_2$ (buffer A), and incubated for 60 minutes at 20°C. The mixture (100 μL) then was centrifuged at 100,000*g* at 4°C with an airfuge (Beckman, Fullerton, Calif). An SDS sample buffer (100 μL) was added to the supernatant. The pellet was resuspended in 100 μL of buffer A and 100 μL of SDS sample buffer. The samples were boiled for 5 minutes and separated on SDS-PAGE (10%) gels. This protocol separated the filamentous actin, which was recovered in the pellet, from the globular actin, which was recovered in the supernatant.

Molecular sieving columns. Strips of bovine carotid artery smooth muscle were equilibrated in bicarbonate buffer and treated with buffer alone (control) or with forskolin (10 μmol/L for 10 minutes). The strips were homogenized (0.5 g tissue/1 mL buffer) in 50 mmol/L Tris, 5 mmol/L EDTA, and pH 7.0 (Tris buffer) with a polytron homogenizer (Brinkman Instruments, Westbury, NY). The samples were centrifuged at 10,000*g*. A total of 500 μL of the supernatant was applied to a Centricon-100 tube (Amicon, Beverly, Mass) and centrifuged at 1000*g* until the upper volume was less than 100 μL. Both the upper and lower solutions were reconstituted to a total volume of 500 μL with Tris buffer. The samples were mixed 1:1 with the sample buffer (10 mmol/L ethyleneglycol-bis(β-aminoethylether)-N,N,N',N'-tetra-acetic acid, 2 mmol/L EDTA, 10 mmol/L β-mercaptoethanol, 1% glycerol, and 4% SDS in 60 mmol/L Tris, pH 7.0) and separated on 15% SDS-PAGE mini gels. The proteins were transferred to Immobilon and probed with anti-HSP20 antibodies followed by enhanced chemiluminescence reagent.

Statistical analysis. The contractile responses were normalized to an initial high KCl contraction, and the results are reported as a percentage of the KCl

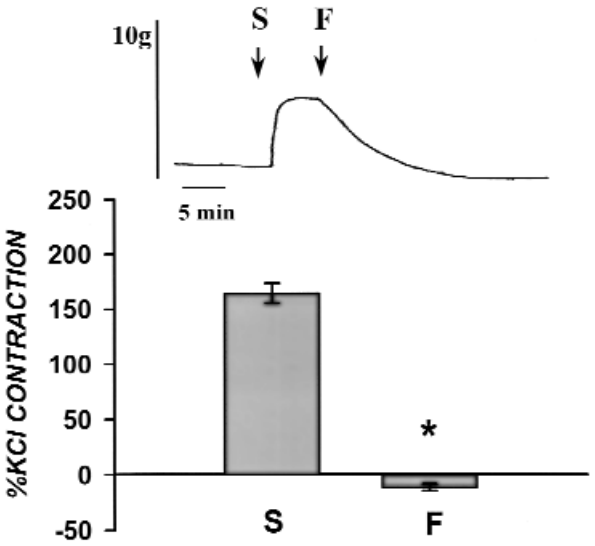

Fig 1. Physiologic contractile responses of bovine carotid artery smooth muscle. Strips of bovine carotid artery smooth muscle were equilibrated in muscle bath, and, after initial high KCl contraction (110 mmol/L), strips were treated with serotonin (10 μmol/L) followed by forskolin (10 μmol/L). *Top panel* is representative tracing in which magnitude of contraction is indicated on *y axis* and time on *x axis*. In *lower panel*, tension was normalized to initial high KCl contraction. Forskolin led to a significant decrease in the contractile response (n = 10).
S, Serotonin; *F,* forskolin.
**P* < .05, with analysis of variance.

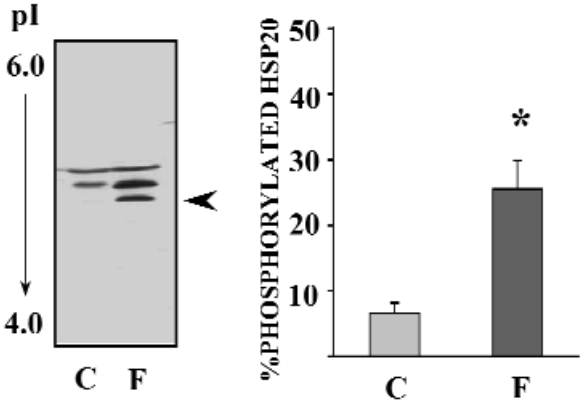

Fig 2. HSP20 phosphorylation. Strips of bovine carotid artery smooth muscle were equilibrated in physiologic bicarbonate solution and then treated with forskolin (10 μmol/L). Proteins were separated on isoelectric-focusing slab gels, transferred to Immobilon, and probed with anti-HSP20 antibodies. Representative immunoblot is on *left panel*, with most acidic isoform, phosphorylated HSP20, identified with *arrow*. Quantitative analysis showed that treatment with forskolin led to significant increase in most acidic isoform of HSP20 (n = 4, *right panel*).
C, Control; *F,* forskolin.
**P* < .05, with analysis of variance.

contraction. The isoelectric-focusing immunoblots were quantitated with a PhosphorImager with ImageQuant software (Molecular Dynamics, Sunnyvale, Calif). The molecular sieving immunoblots were quantitated with a Shimadzu CS-9000 Diamond Scanner (Shimadzu Corp, Japan). Statistical analysis was performed with analysis of variance and Jandel Scientific Software (San Rafael, Calif). The results are presented as the mean ± the standard error of the mean.

RESULTS

Activation of the cAMP/PKA pathway completely relaxes carotid artery smooth muscle. Strips of bovine carotid arteries, devoid of endothelium, were precontracted with serotonin (10 μmol/L). Serotonin-induced contractions were 162.2% ± 9.3% greater in magnitude than the initial high extracellular KCl (110 mmol/L) contraction (Fig 1). The addition of the adenylate cyclase activator, forskolin (10 μmol/L) relaxed the muscles to a level below the baseline (-11.4% ± 3.1% KCl; Fig 1).

Activation of the cAMP/PKA pathway leads to an increase in the phosphorylation of HSP20. In previous studies, we have demonstrated that HSP20 shifts from a basic isoelectric-focusing point to two more acidic isoforms with phosphorylation.[12] With this property, we developed a technique to quantitate HSP20 phosphorylation. The phosphorylated and nonphosphorylated isoforms of HSP20 were separated with isoelectric-focusing slab gels. The proteins then were transferred to Immobilon and probed with affinity purified antibodies that recognize both the phosphorylated and the nonphosphorylated isoforms of HSP20.[13] Treatment with forskolin (10 μmol/L) led to a significant increase in the most acidic isoform of HSP20 (25.5% ± 4.4% compared with the control values of 6.6% ± 1.6%; $P < .05$; n = 4; Fig 2).

Actin coimmunoprecipitates with HSP20. To determine whether HSP20 was associated with the cytoskeletal or the contractile elements of the smooth muscle, HSP20 was immunoprecipitated from the homogenates of the carotid artery smooth muscle. The immunoprecipitated proteins were separated on SDS-PAGE gels, transferred to Immobilon, and

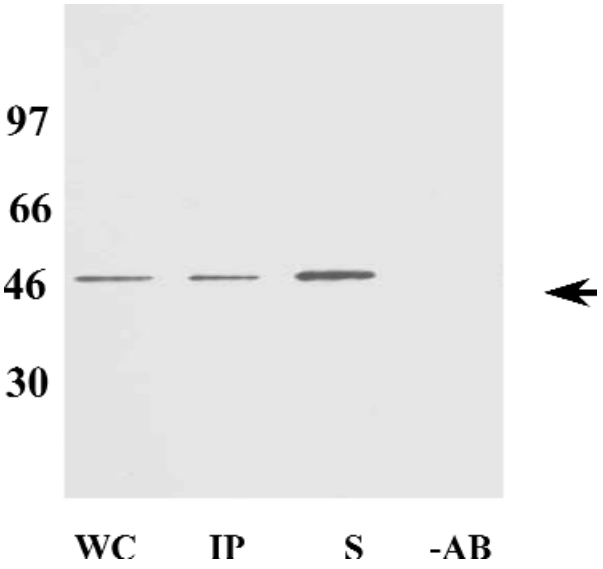

Fig 3. Actin coimmunoprecipitates with HSP20. Strips of bovine carotid arteries were homogenized and 10,000*g* supernatants were immunoprecipitated with anti-HSP20 antibodies. Proteins were separated on SDS-PAGE gels and probed with anti-actin antibodies. The relative mobility of molecular weight markers is indicated on *left* of panel. *WC,* Preimmunoprecipitation fractions; *IP,* immunoprecipitated proteins; *S,* supernatant after immunoprecipitation; *–AB,* immunoprecipitation without the HSP20 antibodies.

probed with anti-actin antibodies. Anti-HSP20 antibodies immunoprecipitated HSP20 (data not shown) and actin (Fig 3).

Phosphorylated HSP20 associates with globular actin, and nonphosphorylated HSP20 with filamentous actin. To determine whether the association of HSP20 with actin was phosphorylation state specific, recombinant HSP20 was expressed in bacteria and phosphorylated in vitro with the catalytic subunit of PKA. The binding of phosphorylated and nonphosphorylated HSP20 to smooth muscle actin was evaluated by means of a cosedimentation assay.[15] Filamentous actin (pellet) was separated from globular actin (supernatant) with high-speed centrifugation. Phosphorylated HSP20 associated with globular actin (Fig 4), which suggests that the phosphorylation of HSP20 may lead to the dissociation of the molecule from cytoskeletal elements.

HSP20 dissociates from a macromolecular aggregate with forskolin treatment. Other small HSPs have been shown to exist in cultured cells in large macromolecular aggregates, which dissociate with various stimuli.[16] To determine whether HSP20 exists in large aggregates in intact vascular smooth muscle and whether these aggregates dissociate with activation of cyclic nucleotide-dependent signaling pathways, homogenates of carotid arteries were separated on Centricon-100 (Fisher Scientific, Norcross, Ga) columns. Most of the immunoreactive HSP20 was in large aggregates in unstimulated carotid arteries (82.3% ± 3.5% > 100 kDa). Stimulation with forskolin (10 μmol/L) led to a dissociation of these aggregates (55.0% ± 2.5% > 100 kDa; Fig 5).

DISCUSSION

Although it is clear that the activation of either the cAMP/PKA or the cGMP/PKG pathway leads to vasorelaxation,[10,11] less is known about the downstream events that result in the actual dissociation of the contractile elements. Because increases in intracellular Ca^{2+} and myosin light chain phosphorylation have been implicated in initiating a contractile response, many investigators have proposed that the activation of cyclic nucleotide dependent signaling pathways leads to decreases in intracellular Ca^{2+} or in myosin light chain phosphorylation. The phosphorylation of myosin light chain kinase by PKA decreases its sensitivity to activation by Ca^{2+}-calmodulin, which leads to a decrease in the phosphorylation of the myosin light chains.[17] PKA or PKG activation leads to changes in the activities of one or more Ca^{2+} channels or Ca^{2+} pumps, which thereby reduces the intracellular Ca^{2+} concentrations.[18,19] However, we and others have shown that there is no simple correlation between the extent of myosin light chain phosphorylation or the intracellular Ca^{2+} concentrations and the state of contraction or relaxation of vascular smooth muscles.[20-25] These data suggest that mechanisms other than decreases in myosin light chain phosphorylation or intracellular Ca^{2+} concentrations alone cannot account for vasorelaxation.

A major phosphorylation event that occurs with cyclic nucleotide-dependent vasorelaxation is an increase in the phosphorylation of two 20 kDa proteins.[20,26,27] We recently identified these 20 kDa phosphoproteins as different phosphorylated forms of a small HSP, HSP20.[12] In addition, HSP20 can be phosphorylated in vitro by both PKA and PKG.[12] Finally, HSP20 is not phosphorylated in a muscle that is refractory to cyclic nucleotide-dependent vasorelaxation—human umbilical artery smooth muscle.[28] Thus, HSP20 appears to be an important downstream regulator of vasorelaxation that is common to both the PKA and the PKG pathways.

Activation of the cAMP/PKA pathway with forskolin leads to the complete relaxation of bovine

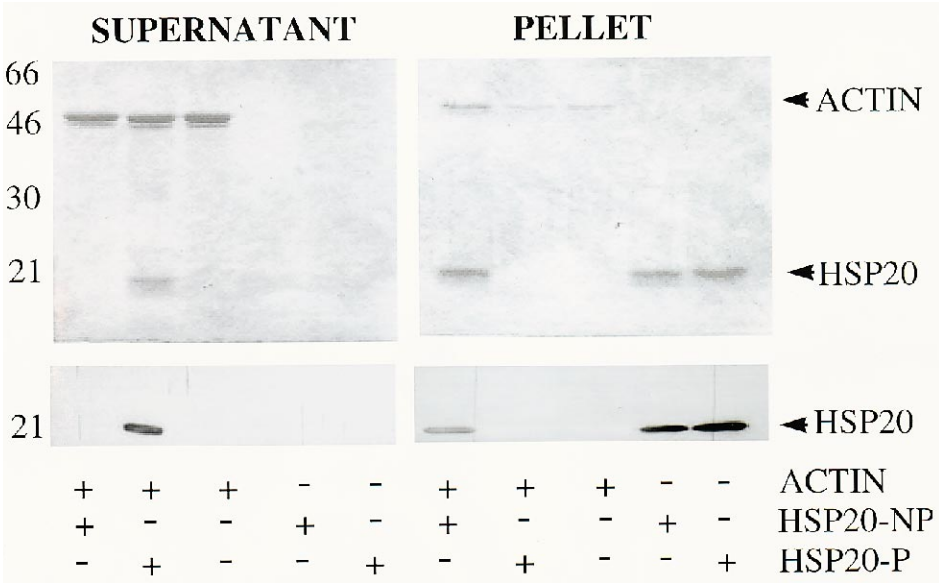

Fig 4. Actin/HSP20 cosedimentation assay. Actin and recombinant HSP20 that were phosphorylated or nonphosphorylated were equilibrated in reaction mixture. The mixture was then centrifuged at 100,000*g*, and supernatant and pellet fractions were separated on SDS-PAGE gels and stained with coomassie (*top panels*). In a separate experiment, proteins were transferred to Immobilon and probed with anti-HSP20 antibodies (*bottom panel*). Relative mobility of molecular weight markers is indicated on the *left* of the panel. Bands with relative mobility of actin (47 kDa) and HSP20 (20 kDa) are indicated on *right*.
HSP20-P, Phosphorylated HSP20; *HSP20-NP,* Nonphosphorylated HSP20.

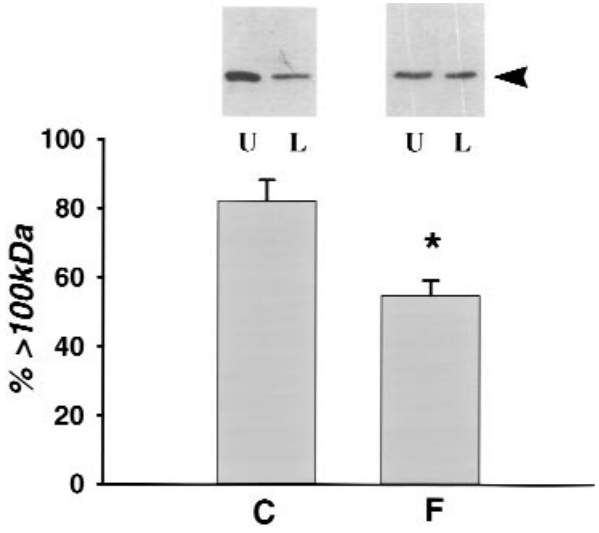

Fig 5. Molecular sieving of HSP20. Strips of bovine carotid arteries were homogenized, and 100,000*g* supernatants were separated on Centricon-100 columns. Immunoblots of proteins in the >100 kDa *(upper)* fraction and the <100 kDa *(lower)* fraction were probed with anti-HSP20 antibodies *(upper panels)*. Percentage of immunoreactive HSP20 in the >100 kDa fraction was determined with densitometry *(bottom panel)*. HSP20 existed in large macromolecular aggregates in unstimulated arteries, which dissociated with forskolin (10 μmol/L) treatment (n = 3).
* $P < .05$, with analysis of variance.
U, Upper; *L,* lower; *C,* unstimulated arteries; *F,* forskolin.

carotid artery smooth muscle (Fig 1). This relaxation is associated with an increase in the more acidic, phosphorylated isoform of HSP20 (Fig 2). Although the precise functions of the HSPs are not known, many HSPs act as "molecular chaperones" that assist in the assembly, the disassembly, the stabilization, and the internal transport of intracellular proteins. The association of HSP20 with actin, and in particular the association of phosphorylated HSP20 with nonfilamentous actin (Figs 3 and 4), suggests that HSP20 may be modulating vasorelaxation by a direct association with cytoskeletal or contractile elements, such as actin. Finally, the macromolecular aggregates of HSP20 dissociate with forskolin stimulation. This suggests that the function of HSP20 in vascular smooth muscle may be related to the state of aggregation of the protein.

Another small HSP, HSP27, has been implicated in the regulation of the contraction of rectal sphincter smooth muscle.[29] HSP27 is phosphorylated by a signaling cascade that involves the tyrosine phosphorylation of p38 mitogen activated protein kinase and the activation of mitogen-activated protein kinase-activated protein-2 kinase-2.[30,31] The phosphorylation of HSP27 is increased during thrombin-induced vascular smooth muscle contraction.[31] HSP27 also has been shown to be important in the regulation of actin filament dynamics.[32,33] HSP20 was first identified as a byproduct of the purification of HSP27.[13] Thus, it is possible that HSP20 modulates vascular tone through an interaction with HSP27.

Taken together, these data support a role for the small HSPs in the regulation of smooth muscle physiology. The small HSPs may modulate contractile physiology via an interaction with each other or with specific cytoskeletal or contractile elements, and these interactions may be modulated with phosphorylation of the molecules.

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